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(54) Title: ANTISENSE INHIBITION OF HEPATITIS C VIRUS

(57) Abstract

The invention features antisense oligonucleotides and methods of using these antisense oligonucleotides for inhibiting HCV RNA translation.

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ANTISENSE INHIBITION OF HEPATITIS C VIRUS

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5   U.S. Government under grant numbers CA-35711 and AA-08169
awarded by the National Institute of Health. The
Government has certain rights in the invention.

Background of the Invention

This invention relates to antisense inhibition of
10 translation of Hepatitis C Virus RNA.

Antisense oligonucleotides (oligos) are useful
tools for studying cellular and viral gene function
(Uhlmann et al., Chem. Rev. 90(4):543-584, 1990; Stein et
al., Science 261:1004-1012, 1993). In addition,
15 antisense oligos are considered to be ideal agents for
inhibiting viral replication, as they can be specifically
targeted to viral RNA sequences, and therefore are not
likely to affect host-specific gene expression.
Antisense oligos have been used in cell culture systems
20 to inhibit the replication of a number of viruses,
including HIV (Zamecnik et al., Proc. Natl. Acad. Sci.
USA 83:4143-4146, 1986; Matsukura et al., Proc. Natl.
Acad. Sci. USA 84:7706-7710, 1987; Lisziewicz et al.,
Proc. Natl. Acad. Sci. USA 89:11209-11213, 1992),
25 influenza (Kabanov et al., FEBS Lett. 259:327-330, 1990),
herpes simplex (Smith et al., Proc. Natl. Acad. Sci. USA
83:2787-2791, 1986), vesicular stomatitis (Lemaitre et
al., Proc. Natl. Acad. Sci. USA 84:648-652, 1987), and
hepatitis B viruses (Goodarzi et al., J. Gen. Virol.
30 71:3021-3025, 1990; Blum et al., Lancet 337:1230, 1991;
Wu et al., J. Biol. Chem. 267(18):12436-12439, 1992;
Offensperger et al., EMBO J. 12:1257-1262, 1993). The
inhibitory effects of antisense oligos on viral activity
is highly specific. For example, Lisziewicz et al. found
35 that two overlapping 28 nucleotide antisense oligos
inhibited HIV replication, but with a 20-fold difference

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in efficiency (Lisziewicz et al., Proc. Natl. Acad. Sci.
USA 89:11209-11213, 1992).

Hepatitis C virus (HCV) is the major causative
agent of post-transfusion hepatitis (Kuo et al., Science
5   244:362-364, 1989). Persistent HCV infection often leads
to chronic hepatitis, cirrhosis, and hepatocellular
carcinoma (Kiyosawa et al., Hepatol. 12:671-675, 1990).
Interferon alpha is widely used as an antiviral agent for
treatment of chronic HCV infection (Hoofnagle et al., N.
10 Engl. J. Med. 315:1575-1578, 1986; Yoshioka et al.,
Hepatol. 16:293-299, 1992). However, the antiviral
effects of interferon have often been found to be limited
and transient.

Summary of the Invention

15   The invention features a method for inhibiting
translation of HCV RNA. In this method, HCV RNA is
contacted with an oligo that is substantially
complementary to a portion of the HCV RNA, and contains a
nucleotide sequence selected from the group consisting of
20 SEQ ID NOs: 1-28. An oligo is described herein as being
"substantially complementary" or "antisense" to a given
nucleic acid, if it is capable of hybridizing to the
nucleic acid under physiological conditions. The oligo
used in the method of the invention is preferably between
25 about 10 and about 400 nucleotides in length, more
preferably is less than about 250 nucleotides in length,
more preferably is less than about 100 nucleotides in
length, more preferably is less than about 50 nucleotides
in length, and most preferably is between about 12 and
30 about 28 nucleotides in length. The oligo can be made of
DNA, RNA, or any modifications or combinations thereof
that preserve the oligo's ability to hybridize to its
complement. For example, the oligo can be an
oligodeoxynucleotide, an oligoribonucleotide, a

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phosphorothioate oligonucleotide, or a methylphosphonate
oligonucleotide, or can contain any combination of these,
and other, types of nucleotides and/or inter-nucleotide
linkages. A "phosphorothioate oligo" is defined as an
5 oligo containing one or more phosphorothioate inter-
nucleotide linkages, while a "methylphosphonate oligo" is
defined as an oligo containing one or more
methylphosphonate inter-nucleotide linkages. In both of
these types of oligos, the modified inter-nucleotide
10 linkages can be present in specific regions of the oligo,
e.g., the 5' and/or 3' ends; can be present in random
positions within the oligo; or can be present throughout
the oligo. It is understood that when the oligo of the
invention is a ribonucleotide, "T" in each of the
15 sequences set forth herein represents "U".

        In a preferred embodiment, the oligo contains a
nucleotide sequence selected from the group consisting of
SEQ ID NOs: 2, 6, 7, 14, 16, 17, 20, and 23-26. In
another embodiment, the oligo contains a nucleotide
20 sequence selected from the group consisting of SEQ ID
NOs: 1, 3, 5, 8-13, 15, 18, 19, 21, 22, 27, and 28. In
another preferred embodiment, the nucleotide sequence of
the oligo consists of SEQ ID NO: 2, 6, 7, 14, 16, 17, 20,
23, 24, 25, or 26.

25   The method of the invention can be used to inhibit
translation of types I, II, III, IV, and V HCV RNA. The
method can be carried out *in vivo*, *ex vivo*, or *in vitro*.
The HCV RNA can be in an animal cell, e.g., an
hepatocyte, which can be from a mammal, e.g., a human or
30 a chimpanzee. In a preferred embodiment, the oligo and
the HCV RNA are contacted with one another in a cell
(e.g., a human hepatocyte, or a chimpanzee hepatocyte) by
introducing into the cell a vector containing a sequence
which is transcribed in the cell to produce the oligo as
35 an oligoribonucleotide.

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In another aspect, the invention features a vector containing a nucleotide sequence which is transcribed in an animal cell to generate the oligo of the invention as an oligoribonucleotide. Preferably, the transcribed
5 nucleotide sequence is operably linked to transcription control sequences that function in hepatocytes. Transcription control sequences can include a transcriptional promoter and/or enhancer, and sequences which control the termination of transcription.
10 Transcription control sequences and a transcribed nucleotide sequence are described herein to be "operably linked" if the transcription control sequences control transcription of the transcribed nucleotide sequence. Transcription control sequences that can be used in the
15 vectors of the invention include, but are not limited to, hepatocyte-specific promoters, e.g., the albumin, alpha-fetoprotein, alpha-1-antitrypsin, retinol-binding protein, and asialoglycoprotein receptor promoters. Viral promoters and enhancers, such as those from
20 cytomegalovirus, herpes simplex viruses (types I and II), hepatitis viruses (A, B, and C), and Rous sarcoma virus (RSV; Fang et al., Hepatology 10:781-787, 1989), may also be used in the invention. A "vector" is defined as a replicable nucleic acid construct. Vectors of the
25 invention include, but are not limited to, plasmid vectors and viral vectors. Preferred viral vectors of the invention include, but are not limited to, those derived from replication-defective hepatitis viruses (e.g., HBV and HCV), retroviruses, adenovirus, adeno-
30 associated virus, replication defective herpes simplex viruses, and any modified versions of these vectors.

In a final aspect, the invention features a therapeutic composition that can be used in methods of treating or preventing HCV infection. The therapeutic
35 composition contains the oligo of the invention in a

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pharmaceutically acceptable carrier, e.g., physiological
saline. In a related aspect, the invention features a
therapeutic composition containing a vector which
contains a nucleotide sequence that is transcribed in an
5   animal cell to generate an oligo of the invention as an
oligoribonucleotide, in a pharmaceutically acceptable
carrier.

Other features and advantages of the invention
will be apparent from the following detailed description,
10 the drawings, and the claims.

Brief Description of the Drawings
Fig. 1 is a schematic representation of the
structure and organization of the 5' region of the HCV
genome. The locations of the 4 AUG codons are indicated
15 by downward-pointing arrows. Three small open reading
frames (ORFs 1-3) are represented by the boxes containing
oblique lines, while the ORF encoding the core protein is
indicated by the large open box. The 18S rRNA
complementary sequence is located between ORFs 2 and 3
20 (Brown et al., Nucl. Acids Res. 20:5041-5045, 1992), and
is indicated by the black box. Two IRES regions have
been reported to be present in the 5' NCR, and are
indicated by the smaller open boxes (Tsukiyama-Kohara et
al., J. Virol. 66:1476-1483, 1992; Wang et al., J. Virol.
25 67:3338-3344, 1993). The overlapping oligos of the
invention cover the entire 5' NCR and 5' end of the HCV
core coding region. The horizontal arrows represent the
sense and antisense oligos used in this study. The
arrowheads indicate the 3' ends of the oligos.
30   Fig. 2A is a schematic representation of the
structural organization of the vector used to express HCV
RNA in this study. A T7 promoter and either HCV type II,
or HCV type III, cDNA sequences (nucleotide positions 1
to 1321, or 2 to 1321, respectively) were inserted into

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the pUC19 vector. The numbers represent the
corresponding nucleotide positions of HCV cDNA. The AUG
codon of the HCV core open reading frame is indicated.
P22 is a putative HCV nucleocapsid protein, and gp35 is
5   one of the putative HCV envelope proteins (Hijikata et
al., Proc. Natl. Acad. Sci. USA 88:5547-5551, 1991).

Fig. 2B is an autoradiogram of the *in vitro*
translation products of type II HCV RNA, fractionated by
SDS-PAGE. The primary core-envelope protein product
10   migrates at approximately 34 kilo-Daltons (kD), as
indicated.

Fig. 3A is a table showing the dose-dependent
inhibition of HCV RNA translation by antisense oligo A161
(SEQ ID NO: 6). Sense oligo S120 (SEQ ID NO: 32) was
15   used in this experiment as a negative control. The
oligos were added to the reactions at molar ratios
varying from 1:1 to 100:1, with respect to template HCV
RNA. Percent inhibition of protein synthesis by the two
oligos is indicated.

20   Fig. 3B is a table showing the effects of HCV
antisense oligo A161 (SEQ ID NO: 6) and sense oligo S120
(SEQ ID NO: 32) on luciferase mRNA translation.

Fig. 4 is a graph showing the results of fine
sequence analysis of antisense oligo effects in the A377
25   (SEQ ID NO: 17)-defined region of HCV RNA. The numbers
after the "A"s indicate the corresponding HCV genomic
positions of the 5' ends of the oligos, and the numbers
in parentheses indicate the lengths of the oligos. The
positions and sequence specificities of the oligos are
30   illustrated on the left. The arrowheads indicate the
3' ends of the oligos. Percent inhibition of HCV type II
RNA translation by each of the indicated oligos is shown
on the right.

Fig. 5 is an autoradiogram of cDNA synthesis
35   reactions fractionated on a 6% denaturing (urea)

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polyacrylamide gel. The reactions were carried out using
HCV RNA as the template, and oligos A65 (SEQ ID NO: 2;
lane 1), A103 (SEQ ID NO: 4; lane 2), A377 (SEQ ID NO:
17; lane 3), and A387 (SEQ ID NO: 18; lane 4) as primers.
5 The predominate cDNA products are indicated by the
numbered arrows, and unextended oligo primers are
indicated at the bottom of the gel (ODN).

        Fig. 6 is an autoradiogram showing the stability
of HCV RNA during *in vitro* translation reactions. [α-^{33}P]
10 labeled HCV RNAs were incubated with antisense oligos
A377 (SEQ ID NO: 17; lane 1) and A367 (SEQ ID NO: 16;
lane 2), sense oligo S120 (SEQ ID NO: 32; lane 3), and no
oligo (lanes 4 and 5). The RNA mixtures were incubated
with rabbit reticulocyte lysates (lanes 1-4), and then
15 extracted. Lane 5 shows HCV RNA mixed with rabbit
reticulocyte lysate, and extracted immediately, without a
15 minute incubation at 30°C. Full length HCV RNA (1300
nucleotides) is indicated by "a", while the cleaved HCV
RNA products of approximately 900 and 380 nucleotides are
20 indicated by "b" and "c", respectively.

                    Detailed Description
        The invention provides antisense oligos that can
be used in methods for inhibiting HCV RNA translation.
The antisense oligos of the invention can be comprised of
25 DNA, RNA, or any modifications or combinations thereof.
As an example of the modifications that the oligos may
contain, inter-nucleotide linkages other than
phosphodiester bonds, such as phosphorothioate,
methylphosphonate, methylphosphodiester,
30 phosphorodithioate, phosphoramidate, phosphotriester, or
phosphate ester linkages (Uhlman et al., Chem. Rev.
90(4):544-584, 1990; Anticancer Research 10:1169, 1990),
may be present in the oligos, resulting in their
increased stability. Oligo stability may also be

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increased by incorporating 3'-deoxythymidine or 2'-
substituted nucleotides (substituted with, e.g., alkyl
groups) into the oligos during synthesis, by providing
the oligos as phenylisourea derivatives, or by having
5   other molecules, such as aminoacridine or poly-lysine,
linked to the 3' ends of the oligos (see, e.g.,
Anticancer Research 10:1169-1182, 1990). Modifications
of the RNA and/or DNA nucleotides comprising the oligos
of the invention may be present throughout the oligo, or
10 in selected regions of the oligo, e.g., the 5' and/or 3'
ends. The antisense oligos may also be modified so as to
increase their ability to penetrate the target tissue by,
e.g., coupling the oligos to lipophilic compounds. The
antisense oligos of the invention can be made by any
15 method known in the art, including standard chemical
synthesis, ligation of constituent oligos, and
transcription of DNA encoding the oligos, as described
below.

        Hepatocytes are susceptible to HCV infection, and
20 thus are the preferred cellular targets for the antisense
oligos of the invention. Targeting of antisense oligos
to hepatocytes may be achieved by coupling the oligos to
ligands of hepatocyte-specific receptors. For example,
the oligos can be coupled to asialo-orosomucoid, (poly)L-
25 lysine-asialo-orosomucoid, or any other ligands of the
hepatic asialoglycoprotein receptor (Spiess, Biochemistry
29(43):10009-10018, 1990; Wu et al., J. Biol. Chem.
267(18):12436-12439, 1992; Wu et al., Biotherapy 3:87-95,
1991). Similarly, antisense oligos may be targeted to
30 hepatocytes by being conjugated to monoclonal antibodies
that specifically bind to hepatocyte-specific receptors.
Antisense oligos may also be targeted to hepatocytes
using specific vectors, as described below.

        The antisense oligos of the invention may be
35 provided exogenously to a target hepatocyte.

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Alternatively, the antisense oligo may be produced within
the target cell by transcription of a nucleic acid
molecule comprising a promoter sequence operably linked
to a sequence encoding the antisense oligo. In this
5 method, the nucleic acid molecule is contained within a
non-replicating linear or circular DNA or RNA molecule,
is contained within an autonomously replicating plasmid
or viral vector, or is integrated into the host genome.
Any vector that can transfect a hepatocyte may be used in
10 the methods of the invention. Preferred vectors are
viral vectors, including those derived from replication-
defective hepatitis viruses (e.g., HBV and HCV),
retroviruses (see, e.g., WO89/07136; Rosenberg et al., N.
Eng. J. Med. 323(9):570-578, 1990), adenovirus (see,
15 e.g., Morsey et al., J. Cell. Biochem., Supp. 17E, 1993),
adeno-associated virus (Kotin et al., Proc. Natl. Acad.
Sci. USA 87:2211-2215, 1990), replication defective
herpes simplex viruses (HSV; Lu et al., Abstract, page
66, Abstracts of the Meeting on Gene Therapy, Sept. 22-
20 26, 1992, Cold Spring Harbor Laboratory, Cold Spring
Harbor, New York), and any modified versions of these
vectors. Methods for constructing expression vectors are
well known in the art (see, e.g., Molecular Cloning: A
Laboratory Manual, Sambrook et al., eds., Cold Spring
25 Harbor Laboratory, 2nd Edition, Cold Spring Harbor, New
York, 1989).

Appropriate regulatory sequences can be inserted
into the vectors of the invention using methods known to
those skilled in the art, for example, by homologous
30 recombination (Graham et al., J. Gen. Virol. 36:59-72,
1977), or other appropriate methods (Molecular Cloning: A
Laboratory Manual, Sambrook et al., eds., Cold Spring
Harbor Laboratory, 2nd Edition, Cold Spring Harbor, New
York, 1989). Promoters are inserted into the vectors so
35 that they are operably linked 5' to the nucleic acid

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sequence encoding the antisense oligo. Any promoter that
is capable of directing initiation of transcription in a
eukaryotic cell may be used in the invention. For
example, non-tissue specific promoters, such as the
5 cytomegalovirus (DeBernardi et al., Proc. Natl. Acad.
Sci. USA 88:9257-9261, 1991, and references therein),
mouse metallothionine I gene (Hammer, et al., J. Mol.
Appl. Gen. 1:273-288, 1982), HSV thymidine kinase
(McKnight, Cell 31:355-365 1982), and SV40 early (Benoist
10 et al., Nature 290:304-310, 1981) promoters may be used.
Non-tissue specific promoters may be used in the
invention, as expression of antisense HCV oligos in non-
liver cells directed by the non-tissue specific promoters
should be harmless to the non-liver cells, because of the
15 specificity of the antisense oligos of the invention for
viral sequences. However, preferred promoters for use in
the invention are hepatocyte-specific promoters, the use
of which ensures that the antisense oligos are expressed
primarily in hepatocytes. Preferred hepatocyte-specific
20 promoters include, but are not limited to the albumin,
alpha-fetoprotein, alpha-1-antitrypsin, retinol-binding
protein, and asialoglycoprotein receptor promoters.
Viral promoters and enhancers, such as those from
cytomegalovirus, herpes simplex viruses (types I and II),
25 hepatitis viruses (A, B, and C), and Rous sarcoma virus
(RSV; Fang et al., Hepatology 10:781-787, 1989), may also
be used in the invention.

        The antisense oligos of the invention, and the
recombinant vectors containing nucleic acid sequences
30 encoding them, may be used in therapeutic compositions
for preventing or treating HCV infection. The
therapeutic applications of antisense oligos in general
are described, e.g., in the following review articles:
LeDoan et al., Bull. Cancer 76:849-852, 1989; Dolnick,
35 Biochem. Pharmacol. 40:671-675, 1990; Crooke, Annu. Rev.

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Pharmacol. Toxicol. 32, 329-376, 1992. The therapeutic
compositions of the invention may be used alone or in
admixture, or in chemical combination, with one or more
materials, including other antisense oligos or
5   recombinant vectors, materials that increase the
biological stability of the oligos or the recombinant
vectors, or materials that increase the ability of the
therapeutic compositions to penetrate hepatocytes
selectively. The therapeutic compositions of the
10   invention may be administered in pharmaceutically
acceptable carriers (e.g., physiological saline), which
are selected on the basis of the mode and route of
administration, and standard pharmaceutical practice.
Suitable pharmaceutical carriers, as well as
15   pharmaceutical necessities for use in pharmaceutical
formulations, are described in *Remington's Pharmaceutical
Sciences*, a standard reference text in this field, and in
the USP/NF.

The therapeutic compositions of the invention can
20   be administered in dosages determined to be appropriate
by one skilled in the art. An appropriate dosage is one
which effects a reduction in the disease caused by HCV
infection, and/or one which is effective at preventing
HCV infection. It is expected that the dosages will
25   vary, depending upon the pharmacokinetic and
pharmacodynamic characteristics of the particular agent,
and its mode and route of administration, as well as the
age, weight, and health (including renal and hepatic
function) of the recipient; the nature and extent of the
30   disease; the frequency and duration of the treatment; the
type of, if any, concurrent therapy; and the desired
effect. It is expected that a useful dosage contains
between about 0.1 to 100 mg of active ingredient per
kilogram of body weight. Ordinarily, 0.5 to 50 mg, and
35   preferably, 1 to 10 mg of active ingredient per kilogram

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of body weight per day given in divided doses, or in
sustained release form, is appropriate.

The therapeutic compositions of the invention may
be administered to a patient by any appropriate mode,
5 e.g., parenterally, intraperitoneally, or intravenously,
as determined by one skilled in the art. Alternatively,
it may by necessary to administer the treatment
surgically to the target tissue. The treatments of the
invention may be repeated as needed, as determined by one
10 skilled in the art.

The invention also includes any other methods
which accomplish *in vivo* transfer of nucleic acids into
eukaryotic cells. For example, the nucleic acids may be
packaged into liposomes, non-viral nucleic acid-based
15 vectors, erythrocyte ghosts, or microspheres
(microparticles; see, e.g., U.S. Patent No. 4,789,734;
U.S. Patent No. 4,925,673; U.S. Patent No. 3,625,214;
Gregoriadis, Drug Carriers in Biology and Medicine, pp.
287-341 (Academic Press, 1979)). Further, delivery of
20 antisense oligos may be accomplished by direct injection
of the oligos into target tissues, for example, in a
calcium phosphate precipitate or coupled with lipids.

Experimental Data

MATERIALS AND METHODS

25 Construction and Expression of HCV cDNAs

HCV cDNAs (nucleotide positions 1 to 1321) were
isolated from anti-HCV positive sera taken from two
Japanese individuals (TH and NT; kindly provided by Dr.
Shinichi Kakumu, Nagoya University, Japan) using the
30 polymerase chain reaction (PCR), as described (Yoshioka
et al., Hepatol. 16:293-299, 1992). Amplified cDNA
fragments were inserted into the pUC19 cloning vector.
The nucleotide sequences of 3 clones obtained from each
individual were determined by the di-deoxy chain
35 termination sequencing method. The clones exhibited

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0.3 to 0.6% nucleotide sequence variation within the same
individual, and 21.6 to 21.9% nucleotide sequence
variation between the two individuals. Clones from TH
exhibited 94.2 to 95.1% nucleotide sequence homology to
5 HCV-BK (Takamizawa et al., J. Virol. 65:1105-1113, 1991)
and HCV-J (Kato et al., Proc. Natl. Acad. Sci. USA
87:9524-9528, 1990), which are type II HCV clones. One
clone from TH, designated 2-2, was selected for further
analysis. Clones from NT exhibited 94.2 to 94.5%
10 nucleotide sequence homology to HCV-J6, which is a type
III HCV clone (Okamoto et al., J. Gen. Virol. 72:2697-
2704, 1991). A clone designated 4-1 was selected from
patient NT for further study. Bacteriophage T7 promoter
sequences were inserted upstream of the 5' ends of the
15 HCV inserts (Fig. 2), and the resulting expression
vectors, designated pTH for the type II HCV genotype, and
pNT for the type III HCV genotype, were used for further
experiments.

Design and synthesis of oligos

20   A series of sense and antisense
oligodeoxynucleotides were synthesized based on the
sequence of pTH type II HCV by standard phosphoramidite
chemistry using a Milligen/Biosearch 8750 synthesizer.
The oligos were purified after NH_4OH detachment 55°C (6
25 hours) and NAP 25 column (Pharmacia, Piscataway, NJ)
desalting with 0.1 M $NaHCO_3$ by reverse phase HPLC
(Tritylon, TEAA 0.1 M pH 7.25) and an acetonitrile
gradient. The oligos were lyophilized, de-blocked with 1
M acetic acid for 1 hour, neutralized with TEA, passed
30 through a NAP 10 column, and lyophilized to dryness. The
HCV nucleotide positions to which the oligos used in this
study correspond are shown in Fig. 1. In combination,
the antisense oligos are complementary to the entire HCV
5' NCR, and part of the HCV core region. The number
35 given to each oligo indicates the position on the HCV

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genomic map to which the 5' end of the oligo corresponds.
The numbers in parentheses, as shown in Fig. 4, indicate
the lengths of the oligos. The sizes and G-C contents of
the oligos are shown in Table I. The molar
5 concentrations of the oligos used in the experiments were
adjusted according to sequence lengths.

In vitro transcription and translation of HCV RNA

pTH and pNT were linearized using HindIII,
digested with proteinase K, extracted with
10 phenol/chloroform, and precipitated in ethanol, prior to
in vitro transcription. Transcription reactions were
carried out using T7 RNA polymerase (Promega, Madison,
WI), and the resulting transcripts were purified by urea-
polyacrylamide gel electrophoresis (urea-PAGE), followed
15 by precipitation in ethanol. RNA transcripts were
labeled with [α-^{33}P]UTP (New England Nuclear, Billerica,
MA) during the transcription reactions, as required.
Type II HCV RNA was used in this study, unless otherwise
indicated.

20   One μg of HCV RNA was mixed with a 10-fold molar
excess of sense or antisense oligo, or with distilled
water. The mixtures were heated at 70°C for 5 minutes,
and then at 50°C for 10 minutes, followed by incubation
at room temperature for 10 minutes, in order to complete
25 annealing of target HCV RNA and the oligo. In some
experiments, the annealing step was omitted. HCV RNA
incubated in the absence of oligo, and rabbit
reticulocyte lysate incubated without HCV RNA, were used
as controls. In vitro translation reactions were carried
30 out as described (Pelham et al., Eur. J. Biochem. 67:247-
256, 1976), with minor modifications. Rabbit
reticulocyte lysates (Promega, Madison, WI) were added to
HCV RNA, or HCV RNA-oligo hybrids, to a final volume of
25 μl in the presence of [^{35}S]-methionine (New England
35 Nuclear, Billerica, MA). After incubation at 30°C for 1

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hour, 4 μl of each of the reaction mixtures were analyzed
by sodium dodecyl sulfate-polyacrylamide gel
electrophoresis (SDS-PAGE). The gels were dried, and
exposed to X-ray film, and the developed films were
5 analyzed by densitometric scanning.

Two μl of each reaction mixture were precipitated
with TCA. The specific incorporation of radioactivity
into the translated protein products was determined using
a liquid scintillation counter (Pelham et al., Eur. J.
10 Biochem. 67:247-256, 1976). Typically, between 500-
5000 cpm/μl were detected in the translated protein
products. Background counts were around 500 cpm/μl in
the control mixture containing no HCV RNA. The amounts
of HCV core envelope fusion protein produced in control
15 reactions were used as standards, and the amounts of HCV
protein synthesized in the presence of the oligos were
compared after subtraction of background counts. The
percent inhibition of HCV RNA translation by each oligo
was calculated using this data.

20 cDNA synthesis from HCV RNA

Oligos A65, A103, A377, and A387 (SEQ ID NOs: 2,
4, 17, and 18, respectively) were end-labeled with [γ^{33}P]-
ATP (New England Nuclear, Billerica, MA) using T4
polynucleotide kinase (Promega, Madison, WI). Specific
25 incorporation of radioactivity into the oligo was
determined after purification through a Sephadex G-25
spin column (Boehringer Mannheim, Germany). The specific
activity of each oligo was normalized by adding an
appropriate amount of unlabeled oligo. The labeled
30 oligos were hybridized with HCV RNA under the same
conditions as described above for the *in vitro*
translation reactions, and the cDNAs were synthesized by
adding Moloney Murine Leukemia Virus reverse
transcriptase (Gibco BRL, Gaithersburg, Maryland). An
35 aliquot of each reaction mixture was fractionated by

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denaturing urea-PAGE. The gel was dried, and the
products were visualized using autoradiography.

HCV RNA stability

Oligos A377 (SEQ ID NO: 17) and A387 (SEQ ID NO:
5 18) were hybridized with [α-^{33}P]-labeled HCV RNA,
incubated under the same conditions as described above,
and added to rabbit reticulocyte lysate in order to
determine the level of RNaseII activity present in the *in
vitro* translation reactions. After an incubation at 30°C
10 for 15 minutes, the RNA was extracted (Chomczynski et
al., Anal. Biochem. 162:156-159, 1987), and aliquots of
the extracted RNA were analyzed by denaturing urea-PAGE.

RESULTS

In vitro **translation of HCV protein**

15   A cell free *in vitro* translation system was used
to express the HCV core envelope fusion protein, and to
determine whether antisense oligos are capable of
inhibiting HCV RNA translation. The expression vector
used in these experiments contains the complete HCV 5'
20 NCR, and a structural coding region containing the entire
core and part of the envelope region of type II and type
III HCV cDNAs (Fig. 2). The RNA transcribed from this
vector contains 980 nucleotides of HCV coding region.
The *in vitro* translated product from type II HCV RNA
25 fractionates as a 34 kD protein by electrophoresis (Fig.
2). That the *in vitro* translated protein product is
related to HCV, was confirmed by immunoprecipitation with
polyclonal anti-HCV antibodies and monoclonal antibodies
raised against recombinant HCV core protein.

30 **Inhibition of HCV RNA translation by antisense oligos**

A series of antisense and sense oligos
corresponding to the HCV 5' NCR were analyzed for their
effects on *in vitro* translation of HCV RNA (Fig. 1).
These experiments were performed in duplicate, and
35 repeated at least twice for each oligo shown in Fig. 1.

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HCV RNA was incubated in a rabbit reticulocyte lysate
system after hybridization with each oligo, and the
amount of protein produced in these reactions was
determined by measuring the levels of radioactivity
5 incorporated into the core envelope fusion protein.
Further quantification was carried out by densitometric
scanning of exposed X-ray films. Table I summarizes the
results of these analyses, showing the effects of each
oligo on translation of both type II and type III HCV
10 RNA. Sense oligos either inhibited HCV RNA translation
only very weakly, or actually stimulated HCV protein
synthesis, compared to reactions lacking oligos. In
contrast, most of the antisense oligos inhibited HCV RNA
translation to significant levels, compared to reactions
15 lacking oligos. For example, six antisense oligos, A65,
A161, A175, A339, A367, and A377 (SEQ ID NOs: 2, 6, 7,
14, 16, and 17, respectively), exhibited more than 50%
inhibition of both type II and type III HCV RNA
translation. The target sequence of A161 (SEQ ID NO: 6)
20 is in a highly conserved domain in the HCV 5' NCR.
However, other antisense oligos that overlap with this
region of HCV RNA exhibited significantly less inhibition
of HCV RNA translation than A161. For example, A147 (SEQ
ID NO: 5) demonstrated only 20.4% inhibition. A367 (SEQ
25 ID NO: 16), which is complementary to the initiation
codon and HCV core coding region, showed the highest
level of inhibition of HCV RNA translation (97.6%) of the
oligos tested. Oligos A367 (SEQ ID NO: 16) and A377 (SEQ
ID NO: 17), which overlap with one another over a
30 17 nucleotide-long region, both showed a substantial
inhibition of both type II and type III HCV core protein
synthesis, even though the pNT HCV type III RNA has a one
nucleotide mutation or mismatch when compared to the A367
(SEQ ID NO: 16) oligo target sequence. However, other
35 overlapping oligos in this region, as illustrated by

- 18 -

oligos A349 (SEQ ID NO: 15) and A387 (SEQ ID NO: 18),
demonstrated only minor inhibitory effects, even though
A349 (SEQ ID NO: 15) is complementary to the core AUG
start codon. Another HCV core coding region antisense

5 oligo, A446 (SEQ ID NO: 19), also showed weak inhibitory
effects. Taken together, these results indicate that the
sequence immediately downstream from the first AUG codon
of the HCV core open reading frame is a good target
sequence for antisense oligo inhibition of HCV RNA

10 translation. A65 (SEQ ID NO: 2) also inhibited HCV RNA
translation to a substantial degree, even though the
region of HCV RNA that this oligo corresponds to is far
upstream from the initiation codon. It is of interest
that oligo A211 (SEQ ID NO: 8) binds to a putative 18S

15 rRNA complementary sequence (Brown et al., Nucl. Acids
Res. 20:5041-5045, 1992), and demonstrated no inhibitory
effect for both type II and type III HCV RNA translation.
The G-C contents and T_ms of the various oligos were not
related to the degree of HCV RNA translation inhibition

20 exhibited by the oligos (Table I).

A dose dependent inhibitory effect on HCV RNA
translation was observed using A161 (SEQ ID NO: 6; Fig.
3). At the highest concentration of A161 used (molar
ratio of oligo:RNA=100:1), HCV protein synthesis was

25 almost completely blocked. It is noteworthy that even at
this high concentration, the sense oligo (S120; SEQ ID
NO: 32) had no inhibitory effect on HCV RNA translation.
Furthermore, as a control, both of these oligos were
incubated with an un-related mRNA encoding luciferase.

30 Neither A161 (SEQ ID NO: 6) nor S120 (SEQ ID NO: 32)
inhibited luciferase mRNA translation at any
concentration employed (Fig. 3). Other antisense oligos
actually were found to stimulate translation of
luciferase mRNA (Table II). This observation is

35 reminiscent of the observation that translation of HCV

- 19 -

RNA is stimulated by certain sense oligos (Table I). In
summary, certain antisense oligos inhibit HCV RNA
translation in a sequence specific manner *in vitro*, even
though the same oligos were found to, if anything,

5   increase the level of translation of an unrelated mRNA.

To determine whether annealing of antisense oligos
to target RNA prior to the *in vitro* translation reaction
is required for effective inhibition of translation,
translation reactions were carried out without the

10 annealing step. Antisense oligos were added just prior
to the HCV RNA translation step, and the degree of
inhibition was determined. In the absence of the
annealing step, oligos showed the same, or higher, levels
of inhibition of HCV RNA translation compared to

15 reactions carried out with the annealing step (Table II).
**Specific inhibition of HCV RNA translation by short
antisense oligos in the A377 (SEQ ID NO: 17) region**

Oligos A367 (SEQ ID NO: 16) and A377 (SEQ ID NO:
17) were the most effective among those tested as

20 inhibitors of HCV type II and type III RNA translation
(Table I). Since there is a mutation at nucleotide
position 350 in type III HCV RNA (Fig. 5), and because
the sequence complementary to this position is contained
within oligo A367, but not in A377, the HCV region

25 defined by oligo A377 (SEQ ID NO: 17) was selected for
further study to determine the specific nucleotide
sequence requirements for inhibition of HCV RNA
translation. Different length oligos derived from the
A377 (SEQ ID NO: 17)-defined region of HCV were tested

30 with type II HCV RNA in the *in vitro* translation assay.
As nucleotides were deleted from the 3' end of the A377
(SEQ ID NO: 17) sequence, the degree of inhibition was
reduced in proportion to the size of the deletion. In
contrast, deletions from the 5' end of A377 (SEQ ID NO:

35 17) increased the inhibitory effect from 78.4% (A377; SEQ

- 20 -

ID NO: 17; 27 nucleotides) to 96.9% with A367 (SEQ ID NO:
16; 17 nucleotides). It is noteworthy that A362 (SEQ ID
NO: 25), which is only 12 nucleotides in length,
significantly inhibited HCV RNA translation (by 80.7%).

5   The inhibition of translation was substantially reduced
using oligos of only 10 nucleotides in length, e.g.,
oligos A362 (SEQ ID NO: 27) and A360 (SEQ ID NO: 28).
These experiments show that the HCV RNA region between
nucleotide positions 351-367, which is highly conserved

10 in both type II and type III HCV clones, is an excellent
viral RNA target for antisense oligo inhibition.
HCV cDNA synthesis with antisense oligos
        Both the binding efficiencies and the nucleotide
sequence specificities of the various antisense oligos

15 may play roles in determining the degree of inhibition of
HCV RNA translation they exhibit. To determine the
relative binding affinities of different antisense oligos
to HCV RNA, cDNA synthesis assays were carried out.
Oligos A65 (SEQ ID NO: 2) and A377 (SEQ ID NO: 17), which

20 inhibit HCV RNA translation, and oligos A103 (SEQ ID NO:
4) and A387 (SEQ ID NO: 18), which are derived from the
same general regions of the HCV genome as oligos A65 (SEQ
ID NO: 2) and A377 (SEQ ID NO: 17), respectively, but
unlike the latter do not inhibit HCV translation, were

25 used in this analysis. The oligos were labeled with [γ-
^{33}P]ATP, and the specific activities were normalized in
all experiments. The same ratio of HCV RNA to oligo
(1:10) used in the translation assays was used in the
cDNA synthesis reactions. Fig. 5 shows the results of

30 these experiments. The predominant cDNA products
fractionated at their expected sizes by urea-PAGE.
Several smaller bands were detected in lanes 3 and 4
(Fig. 5), and were probably generated in part because of
the secondary structure of the template RNA, which blocks

35 elongation of the cDNAs. The patterns of the DNA ladders

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generated in the reaction containing oligo A377 (SEQ ID
NO: 17; Fig. 5, lane 3), which inhibits HCV RNA
translation, and the reaction containing oligo A387 (SEQ
ID NO: 18; Fig. 5, lane 4), which does not inhibit HCV

5   RNA translation, are nearly identical. These data show
that inhibition of HCV RNA translation is not a function
of non-specific binding of oligos to HCV RNA target
sequences, but rather, is due to the specificity of the
HCV RNA target sequence/antisense oligo interaction.

10 Further, these data show that the differences in
inhibition of HCV RNA translation observed using
different antisense oligos is not due to different
binding affinities of the oligos, but rather is due to
binding specificity of the oligos to HCV RNA, as the

15 levels of cDNA products produced in the reactions using
different antisense oligos as primers are similar.

HCV RNA stability

RNase H activity has been proposed to be one of
the major mechanisms for the inhibitory effects of

20 antisense oligos (Uhlmann et al., Chem. Rev. 90(4):543-
584, 1990). To determine the stability of HCV RNA during
in vitro translation reactions, [^{33}P]-labeled HCV RNA was
synthesized and purified by gel electrophoresis. Labeled
HCV RNA was hybridized with oligos, and incubated with

25 rabbit reticulocyte lysates in the translation assay.
After 15 minutes of incubation at 30°C, HCV RNA was
extracted, and loaded onto gels under denaturing
conditions. HCV RNA incubated without any oligo showed
the expected full size transcripts, and minor degradation

30 products (Fig 6, lanes 4 and 5). However, RNAs
hybridized with oligos demonstrated two additional major
bands (Fig. 6, lanes 1 and 2). The sizes of the shorter
RNA bands are consistent with their corresponding to
cleaved products generated by RNase H activity. It is

35 noteworthy that labeled HCV RNAs were not completely

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degraded under these conditions. Furthermore, similar
levels of HCV RNA cleavage were observed for both
inhibitory and non-inhibitory oligos. Thus, it is not
likely that RNAse H degradation is the major mechanism
5   whereby the above-described antisense oligos inhibit HCV
RNA translation.

Other embodiments

        From the above description, one skilled in the art
can easily ascertain the essential characteristics of the
10 present invention, and without departing from the spirit
and scope thereof, can make various changes and
modifications of the invention to adapt it to various
usages and conditions. It is understood that oligos
having minor variations from the disclosed sequences
15 which do not affect the biological function (i.e., the
ability of the oligo to hybridize to, and to inhibit the
translation of, HCV RNA) of the oligos are within the
invention.

        All publications cited herein are fully
20 incorporated by reference herein in their entirety.

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Table I. Percent Inhibition of Type II and III HCV Translation by oligos

			Oligos[a]	Size (nt)	GC (%)	Inhibition of protein synthesis [b] (%)	
						Type II	Type III
SEQ ID NO:	1	A37	28	57	5	ND	
SEQ ID NO:	2	A65	28	54	74	54	
SEQ ID NO:	3	A81	28	46	40	16 (1)[c]	
SEQ ID NO:	4	A103	28	50	-1	-1 (1)	
SEQ ID NO:	5	A147	28	71	20	14	
SEQ ID NO:	6	A161	28	61	81	76	
SEQ ID NO:	7	A175	28	57	55	88	
SEQ ID NO:	8	A211	28	61	4	-20	
SEQ ID NO:	9	A242	28	68	34	ND	
SEQ ID NO:	10	A270	28	57	44	ND	
SEQ ID NO:	11	A298	28	54	40	35	
SEQ ID NO:	12	A312	28	57	45	18	
SEQ ID NO:	13	A326	28	71	64	30	
SEQ ID NO:	14	A339	27	70	53	50	
SEQ ID NO:	15	A349	28	61	41	39 (1)	
SEQ ID NO:	16	A367	27	44	98	95 (1)	
SEQ ID NO:	17	A377	27	30	80	94	
SEQ ID NO:	18	A387	27	37	15	22 (2)	
SEQ ID NO:	19	A446	27	52	13	4 (3)	
SEQ ID NO:	29	S38	28	54	-4	ND	
SEQ ID NO:	30	S54	28	46	0	ND	
SEQ ID NO:	31	S76	28	50	2	-15	
SEQ ID NO:	32	S120	28	71	-21	-2	
SEQ ID NO:	33	S148	28	57	-11	29	
SEQ ID NO:	34	S271	28	54	-30	-33	
SEQ ID NO:	35	S291	28	68	-37	-33	
SEQ ID NO:	36	S312	28	68	-35	-25	

[a]A = Antisense oligo, S=sense oligo. The number after A or S indicates the position of the 5' end of the oligo. The underlining indicates oligos that inhibit HCV type II and III translation by greater than 50%.
[b]Inhibition assays were performed as described in materials and methods. Each oligo was used at 10 fold molar excess relative to HCV RNA. ND=not determined.
[c]The numbers in parentheses indicate the mutations in the target sequence of the type III clone.

Table II. Inhibition of HCV and Luciferase RNA translation by antisense oligos, and effect of the annealing step on the inhibition of HCV RNA translation

		Inhibition of translation (%)		Luciferase[a]
		annealing(+)	annealing(-)	
SEQ ID NO: 2	A65	75	73	-2
SEQ ID NO: 6	A161	77	83	-27
SEQ ID NO: 14	A339	60	63	-84
SEQ ID NO: 17	A377	84	93	-49

[a] All _in vitro_ translations with Luciferase RNA were performed with annealing steps

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<u>SEQUENCE LISTING</u>

(1) GENERAL INFORMATION:

    (i) APPLICANT: Wakita, Takaji
                                  Wands, Jack

    (ii) TITLE OF INVENTION: ANTISENSE INHIBITION OF
                                  HEPATITIS C VIRUS

    (iii) NUMBER OF SEQUENCES: 38

    (iv) CORRESPONDENCE ADDRESS:

        (A) ADDRESSEE: Fish & Richardson
        (B) STREET: 225 Franklin Street
        (C) CITY: Boston
        (D) STATE: Massachusetts
        (E) COUNTRY: U.S.A.
        (F) ZIP: 02110-2804

    (v) COMPUTER READABLE FORM:

        (A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
        (B) COMPUTER: IBM PS/2 Model 50Z or 55SX
        (C) OPERATING SYSTEM: MS-DOS (Version 5.0)
        (D) SOFTWARE: WordPerfect (Version 5.1)

    (vi) CURRENT APPLICATION DATA:

        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 08/240,382
        (B) FILING DATE: 10 May 1994

    (viii) ATTORNEY/AGENT INFORMATION:

        (A) NAME: Clark, Paul T.
        (B) REGISTRATION NUMBER: 30,162
        (C) REFERENCE/DOCKET NUMBER:00786/221001

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        (C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

    (i) SEQUENCE CHARACTERISTICS:

- 26 -

```
        (A) LENGTH:              28
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear
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   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATCTATGGTG GAGTGTCGCC CCCAATCG   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

   (i) SEQUENCE CHARACTERISTICS:

```
        (A) LENGTH:              28
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear
```

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAAGACAGTA GTTCCTCACA GGGGAGTG   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

   (i) SEQUENCE CHARACTERISTICS:

```
        (A) LENGTH:              28
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear
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   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TAGACGCTTT CTGCGTGAAG ACAGTAGT   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

   (i) SEQUENCE CHARACTERISTICS:

```
        (A) LENGTH:              28
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear
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   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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ACACTCATAC TAACGCCATG GCTAGACG   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 28
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ACCACTATGG CTCTCCCGGG AGGGGGGG   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 28
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CACCGGTTCC GCAGACCACT ATGGCTCT   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 28
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AATTCCGGTG TACTCACCGG TTCCGCAG   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 28
        (B) TYPE: nucleic acid

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```
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:


GCGGGTTGAT CCAAGAAAGG ACCCGGTC                    28


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    9:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:              28
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:


GCGGGGGCAC GCCCAAATCT CCAGGCAT                    28


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    10:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:              28
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:


GACCCAACAC TACTCGGCTA GCAGTCTC                    28


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:    11:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:              28
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:


TATCAGGCAG TACCACAAGG CCTTTCGC                    28
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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 12:

   (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 28
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ACTCGCAAGC ACCCTATCAG GCAGTACC   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 13:

   (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 28
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AGACCTCCCG GGGCACTCGC AAGCACCC   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 14:

   (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 28
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TGCACGGTCT ACGAGACCTC CCGGGGCA   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 15:

   (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 28
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

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GTGCTCATGG TGCACGGTCT ACGAGACC   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 16:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 27
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CTTTGAGGTT TAGGATTCGT GCTCATG   27

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 17:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 27
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TTTGGTTTTT CTTTGAGGTT TAGGATT   27

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 18:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 27
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TGGTGTTACG TTTGGTTTTT CTTTGAG   27

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 19:

   (i) SEQUENCE CHARACTERISTICS:

- 31 -

```
              (A) LENGTH:              27
              (B) TYPE:                nucleic acid
              (C) STRANDEDNESS:        single
              (D) TOPOLOGY:            linear
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   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GTAAACTCCA CCAACGATCT GACCACC   27

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 20:

   (i) SEQUENCE CHARACTERISTICS:

```
              (A) LENGTH:              22
              (B) TYPE:                nucleic acid
              (C) STRANDEDNESS:        single
              (D) TOPOLOGY:            linear
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   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

TTTGGTTTTT CTTTGAGGTT TA   22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 21:

   (i) SEQUENCE CHARACTERISTICS:

```
              (A) LENGTH:              17
              (B) TYPE:                nucleic acid
              (C) STRANDEDNESS:        single
              (D) TOPOLOGY:            linear
```

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

TTTGGTTTTT CTTTGAG   17

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 22:

   (i) SEQUENCE CHARACTERISTICS:

```
              (A) LENGTH:              12
              (B) TYPE:                nucleic acid
              (C) STRANDEDNESS:        single
              (D) TOPOLOGY:            linear
```

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

TTTGGTTTTT CT   12

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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 23:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 22
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

TTTTTCTTTG AGGTTTAGGA TT   22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 24:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 17
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CTTTGAGGTT TAGGATT   17

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 25:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 12
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

AGGTTTAGGA TT   12

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 26:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 12
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CTTTGAGGTT TA   12

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 27:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 10
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

AGGTTTAGGA   10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 28:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 10
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GTTTAGGATT   10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 29:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 28
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CACTCCCCTG TGAGGAACTA CTGTCTTC   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 30:

    (i) SEQUENCE CHARACTERISTICS:

- 34 -

```
        (A) LENGTH:              28
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear
```

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

ACTACTGTCT TCACGCAGAA AGCGTCTA   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 31:

   (i) SEQUENCE CHARACTERISTICS:

```
        (A) LENGTH:              28
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear
```

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CGTCTAGCCA TGGCGTTAGT ATGAGTGT   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 32:

   (i) SEQUENCE CHARACTERISTICS:

```
        (A) LENGTH:              28
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear
```

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

CCCCCCCTCC CGGGAGAGCC ATAGTGGT   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 33:

   (i) SEQUENCE CHARACTERISTICS:

```
        (A) LENGTH:              28
        (B) TYPE:                nucleic acid
        (C) STRANDEDNESS:        single
        (D) TOPOLOGY:            linear
```

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

- 35 -

CTGCGGAACC GGTGAGTACA CCGGAATT   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 34:

   (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 28
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GCGAAAGGCC TTGTGGTACT GCCTGATA   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 35:

   (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 28
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

GCCTGATAGG GTGCTTGCGA GTGCCCCG   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 36:

   (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 28
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

TGCCCCGGGA GGTCTCGTAG ACCGTGCA   28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 37:

   (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 29
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single

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    (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

CGAAUCCUAA ACCUCAAAGA AAAACCAAA   29

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 38:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 29
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

CAAAUCCUAA ACCUCAAAGA AAAACCAAA   29

    What is claimed is:

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1. A method of inhibiting translation of
Hepatitis C Virus RNA, said method comprising contacting
said RNA with an oligonucleotide substantially
complementary to a portion of said RNA, said

5   oligonucleotide being an RNA or a DNA molecule comprising
a sequence selected from the group consisting of SEQ ID
NOs: 1-28, said oligonucleotide being between about 10
and about 400 nucleotides in length.

2. The method of claim 1, wherein said

10 oligonucleotide is less than about 250 nucleotides in
length.

3. The method of claim 2, wherein said
oligonucleotide is less than about 100 nucleotides in
length.

15 4. The method of claim 3, wherein said
oligonucleotide is less than about 50 nucleotides in
length.

5. The method of claim 4, wherein said
oligonucleotide is between about 12 and about 28

20 nucleotides in length.

6. The method of claim 1, wherein said Hepatitis
C Virus is selected from the group consisting of
Hepatitis C Virus types I, II, III, IV, and V.

7. The method of claim 1, wherein said Hepatitis

25 C Virus RNA is in an animal cell.

8. The method of claim 7, wherein said animal
cell is an hepatocyte.

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9.   The method of claim 7, wherein said animal cell is in a mammal.

10.   The method of claim 9, wherein said mammal is a human.

5   11.   The method of claim 7, wherein said contacting is carried out by introducing into said animal cell a vector containing a sequence which is transcribed in said animal cell to produce said oligonucleotide as an oligoribonucleotide.

10   12.   The method of claim 1, wherein said oligonucleotide comprises a sequence selected from the group consisting of SEQ ID NOs: 2, 6, 7, 14, 16, 17, 20, and 23-26.

13.   The method of claim 1, wherein the nucleotide
15 sequence of said oligonucleotide consists of SEQ ID NO: 2.

14.   The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 6.

20   15.   The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 7.

16.   The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO:
25 14.

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17. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 16.

18. The method of claim 1, wherein the nucleotide
5 sequence of said oligonucleotide consists of SEQ ID NO: 17.

19. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 20.

10   20. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 23.

21. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO:
15 24.

22. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 25.

23. The method of claim 1, wherein the nucleotide
20 sequence of said oligonucleotide consists of SEQ ID NO: 26.

24. The method of claim 1, wherein the nucleotide sequence of said oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID
25 NOs: 1, 3, 5, 8-13, 15, 18, 19, 21, 22, 27, and 28.

25. An oligonucleotide substantially complementary to a portion of Hepatitis C Virus RNA, said

oligonucleotide being an RNA or a DNA molecule comprising
a sequence selected from the group consisting of SEQ ID
NOs: 1-28, said oligonucleotide being between about 10
and about 400 nucleotides in length.

5   26.   The oligonucleotide of claim 25, wherein said
oligonucleotide is less than about 250 nucleotides in
length.

          27.   The oligonucleotide of claim 26, wherein said
oligonucleotide is less than about 100 nucleotides in
10   length.

          28.   The oligonucleotide of claim 27, wherein said
oligonucleotide is less than about 50 nucleotides in
length.

          29.   The oligonucleotide of claim 28, wherein said
15   oligonucleotide is between about 12 and about 28
nucleotides in length.

          30.   The oligonucleotide of claim 25, wherein the
nucleotide sequence of said oligonucleotide comprises a
nucleotide sequence selected from the group consisting of
20   SEQ ID NOs: 2, 6, 7, 14, 16, 17, 20, and 23-26.

          31.   The oligonucleotide of claim 25, wherein the
nucleotide sequence of said oligonucleotide consists of
SEQ ID NO: 2.

          32.   The oligonucleotide of claim 25, wherein the
25   nucleotide sequence of said oligonucleotide consists of
SEQ ID NO: 6.

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33. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 7.

34. The oligonucleotide of claim 25, wherein the
5   nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 14.

35. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 16.

10   36. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 17.

37. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of
15   SEQ ID NO: 20.

38. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 23.

39. The oligonucleotide of claim 25, wherein the
20   nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 24.

40. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 25.

25   41. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide consists of SEQ ID NO: 26.

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42. The oligonucleotide of claim 25, wherein the nucleotide sequence of said oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 8-13, 15, 18, 19, 21, 22, 27, and
5   28.

43. A vector comprising a nucleotide sequence which is transcribed in an animal cell to generate the oligonucleotide of claim 23, said oligonucleotide being an oligoribonucleotide.

10   44. The vector of claim 43, wherein said transcribed nucleotide sequence is operably linked to transcription control sequences that function in hepatocytes.

45. The oligonucleotide of claim 25, wherein said
15   oligonucleotide is an oligodeoxynucleotide.

46. The oligonucleotide of claim 25, wherein said oligonucleotide is an oligoribonucleotide.

47. The oligonucleotide of claim 25, wherein said oligonucleotide is a phosphorothioate oligonucleotide.

20   48. The oligonucleotide of claim 25, wherein said oligonucleotide is a methylphosphonate oligonucleotide.

49. A therapeutic composition comprising the oligonucleotide of claim 25 in a pharmaceutically acceptable carrier.

25   50. A therapeutic composition comprising the vector of claim 43 in a pharmaceutically acceptable carrier.
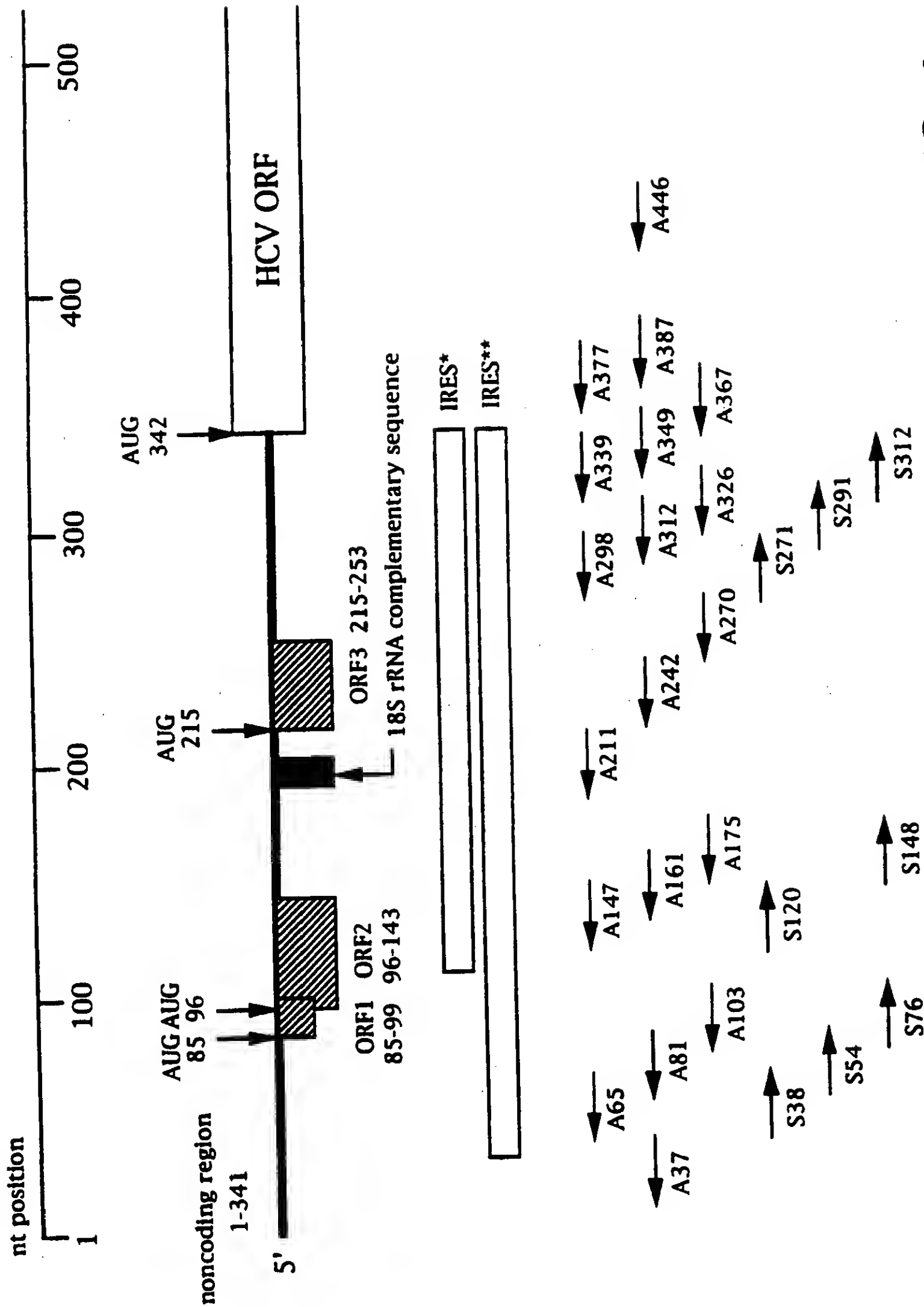
FIG. 1
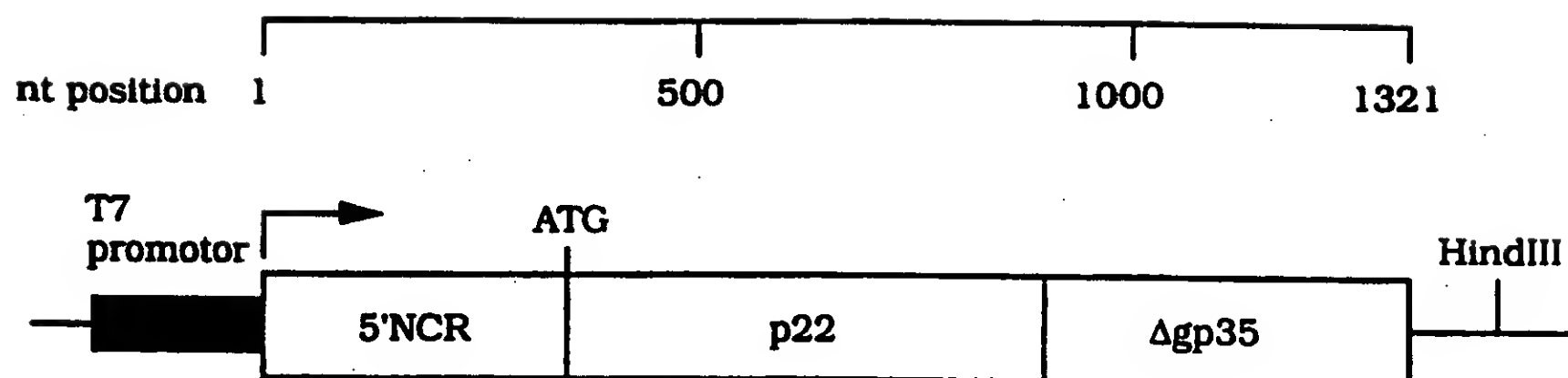
FIG. 2A

FIG. 2B
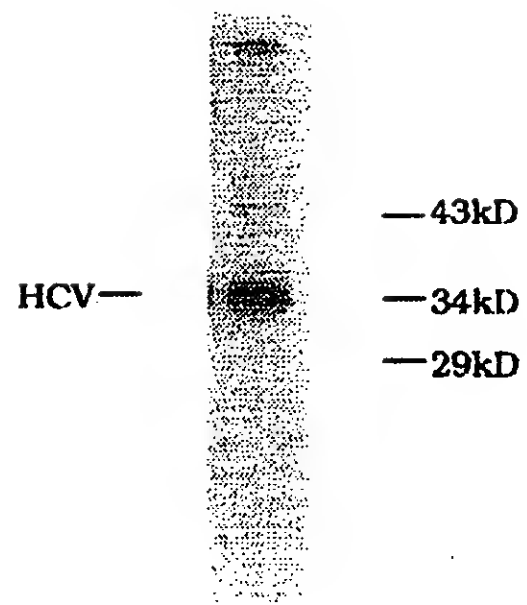

FIG. 6
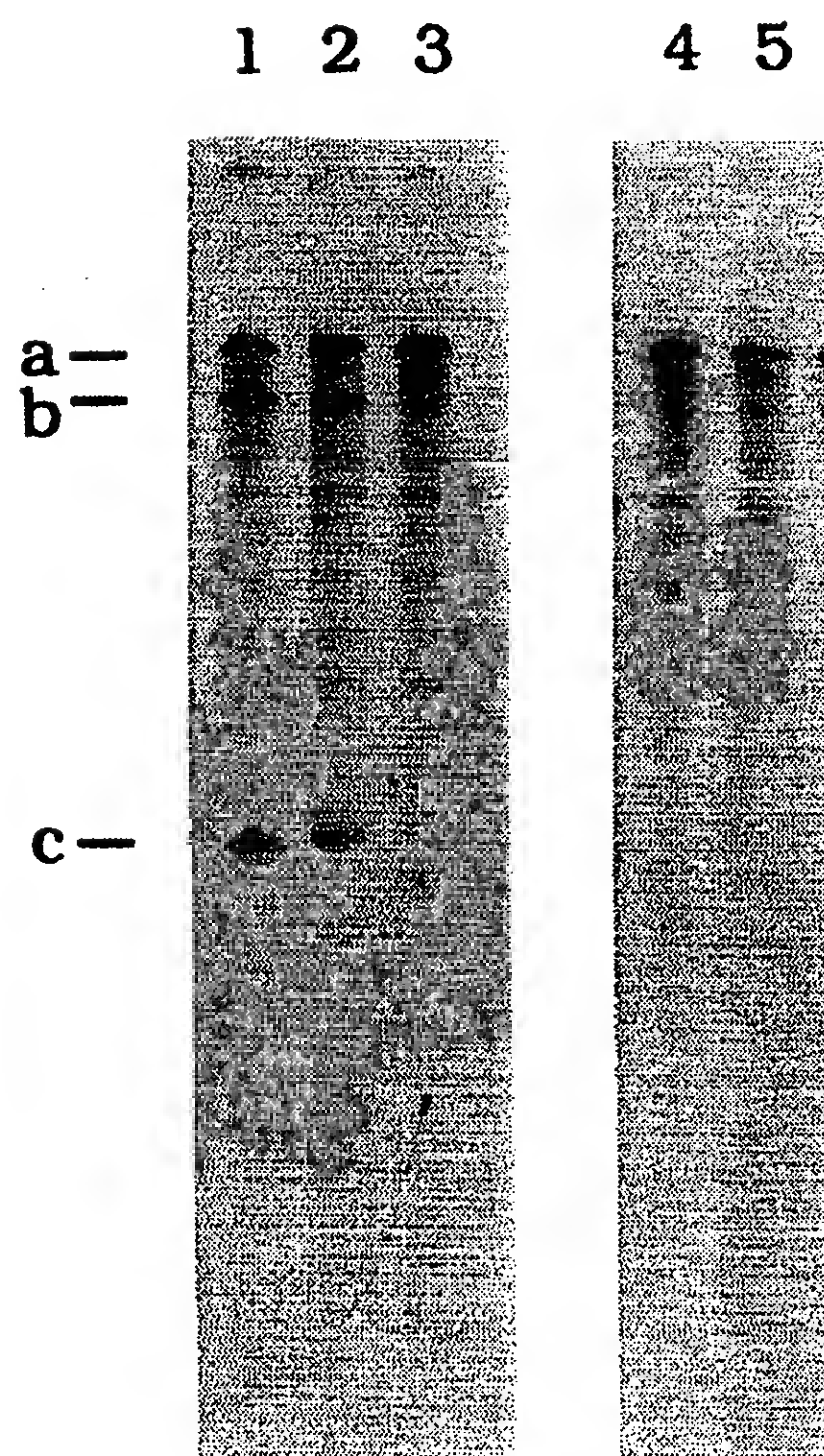SUBSTITUTE SHEET (RULE 26)

FIG. 5
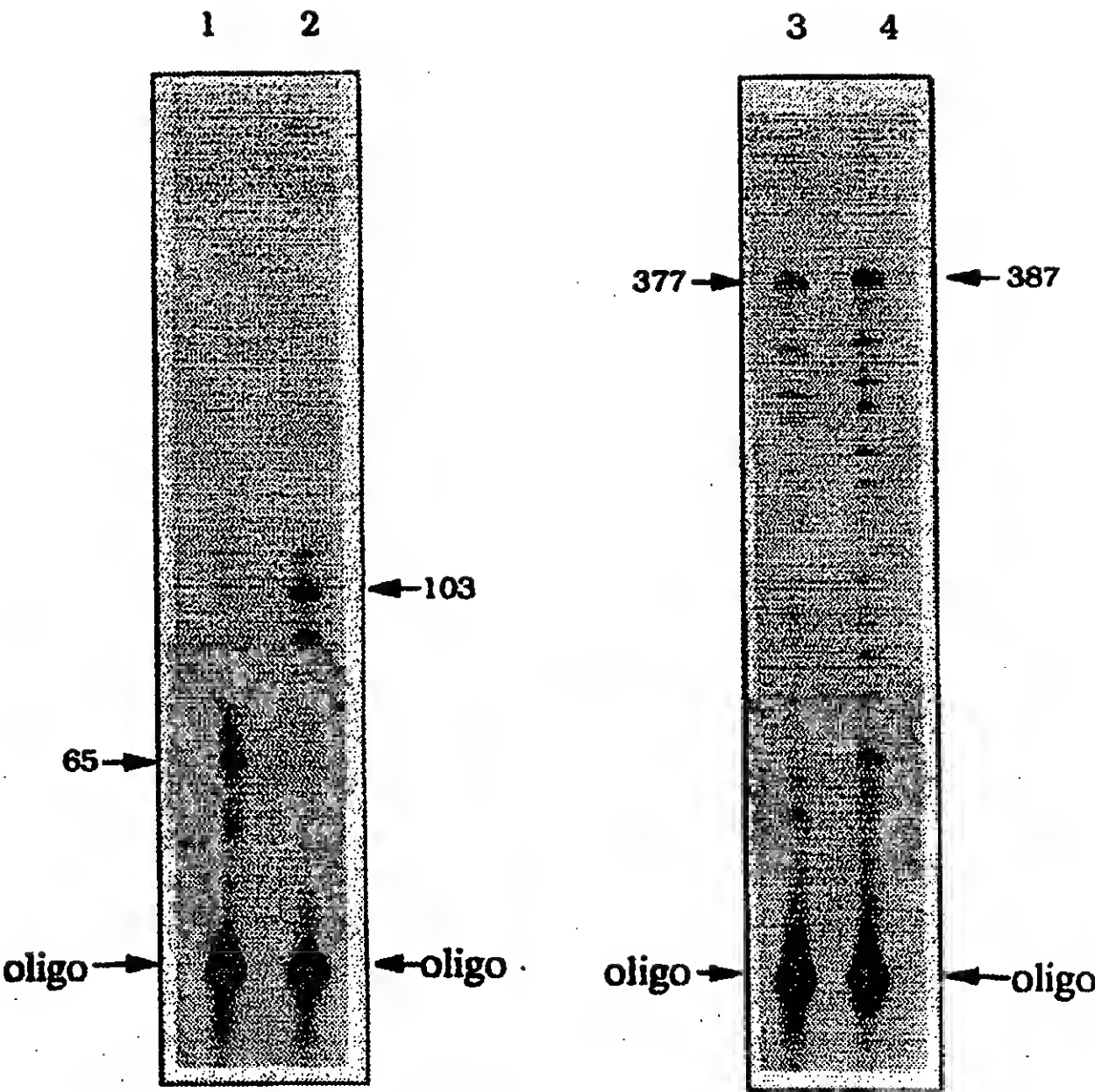
HCV translation

oligo	(-)	A161			S120	
oligo/RNA	0	1	10	100	10	100
HCV —						
Inhibition of protein synthesis(%)	0	18	74	93	-22	-13

FIG. 3A

Luciferase translation

oligo	(-)	A161			S120	
oligo/RNA	0	1	10	100	10	100
Luciferase—						
Inhibition of protein synthesis(%)	0	-7	-21	-1	-17	-46

FIG. 3B
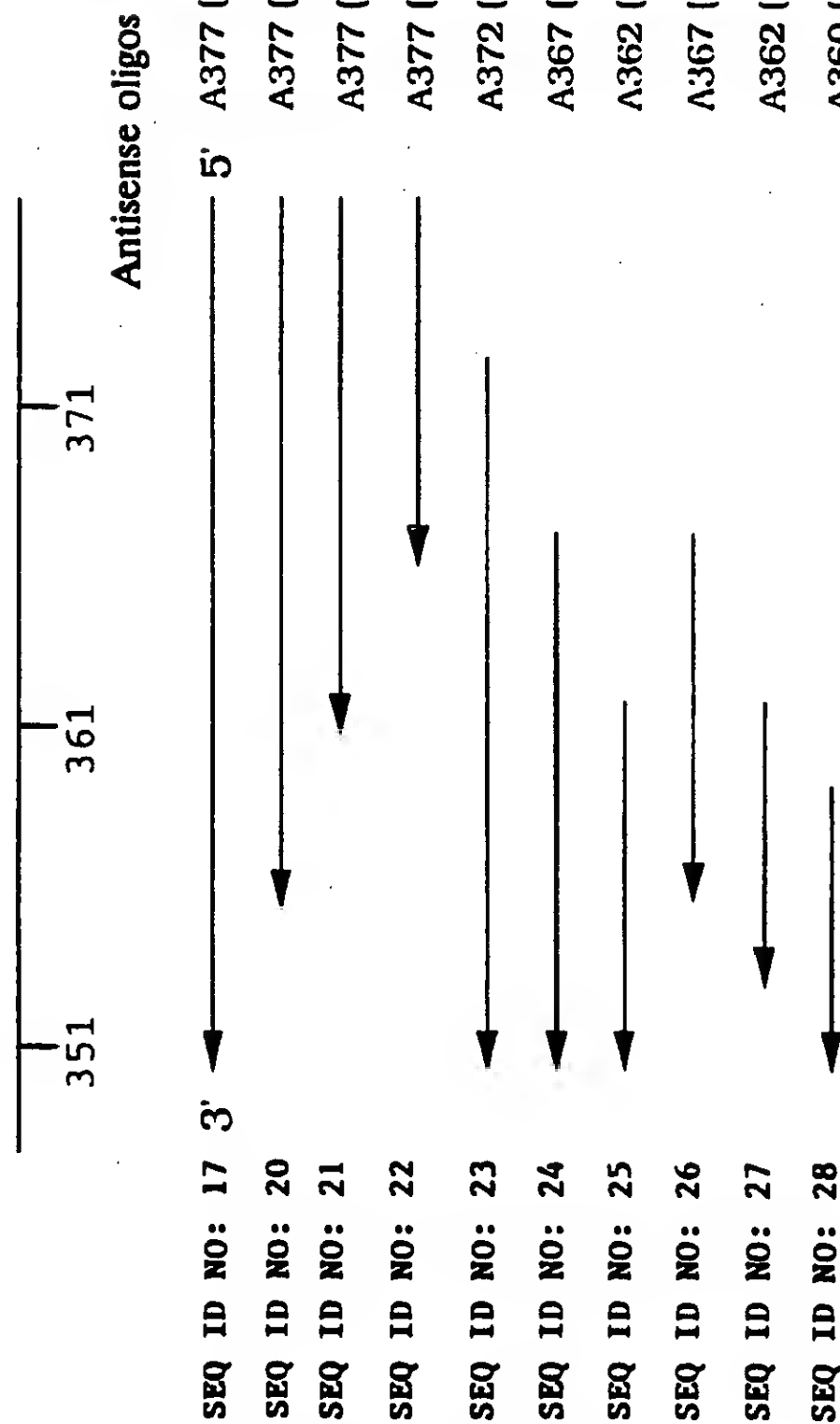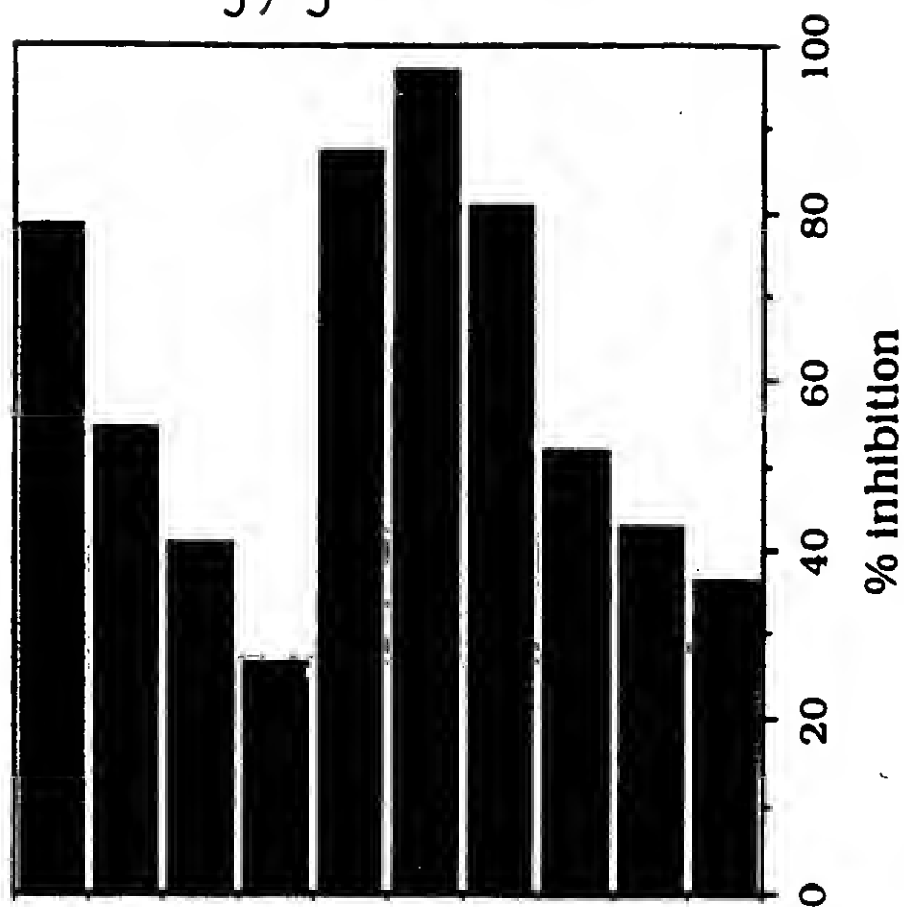
FIG. 4

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/00, 15/11, 15/51; A61K 48/00

US CL :514/44; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline

hepatitis, hepatitis C, oligonucleotide, oligodeoxynucleotide, antisense, antiviral

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Medicinal Chemistry, Volume 36, Number 14, issued 09 July 1993, Milligan et al., "Current Concepts in Antisense Drug Design", pages 1923-1937, see entire document.	1-50
Y	Science, Volume 261, issued 20 August 1993, Stein et al., "Antisense Oligonucleotides as Therapeutic Agents-Is the Bullet Really Magical?", pages 1004-1011, see entire document.	1-50
Y	Journal of Biological Chemistry, Volume 267, Number 18, issued 25 June 1992, Wu et al., "Specific Inhibition of Hepatitis B Viral Gene Expression *in Vitro* by Targeted Antisense Oligonucleotides", pages 12436-12439, see entire document.	1-50

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 JUNE 1995	10 JUL 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MICHAEL J. NEWELL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of General Virology, Volume 71, issued 1990, Goodarzi et al., "Antisense Oligodeoxyribonucleotides Inhibit the Expression of the Gene for Hepatitis B Virus Surface Antigen", pages 3021-3025, see entire document.	1-50
Y	EMBO Journal, Volume 12, Number 3, issued 1993, Offensperger et al., "*In Vivo* Inhibition of Duck Hepatitis B Virus Replication and Gene Expression by Phosphorothioate Modified Antisense Oligodeoxynucleotides", pages 1257-1262, see entire document.	1-50
T	Pharmacology and Therapeutics, Volume 65, issued 1995, Dusheiko, "Treatment and Prevention of Chronic Viral Hepatitis", pages 47-73, see entire document.	1-50
Y,P	US, A, 5,346,696 (KIM ET AL.) 13 September 1994, see entire document.	1-50
Y	Science, Volume 244, issued 21 April 1989, Choo et al. "Isolation of a cDNA Clone Derived from a Blood-Borne Non-A, Non-B Viral Hepatitis Genome", pages 359-362, see entire document.	1-50
Y	Advances in Experimental Medicine and Biology, Volume 342, issued 1993, Mizutani et al., "Inhibition of Mouse Hepatitis Virus Multiplication by Antisense Oligonucleotide, Antisense RNA, Sense RNA and Ribozyme", pages 129-135, see entire document.	1-50
Y,P	US, A, 5,372,928 (MIYAMURA ET AL.) 13 December 1994, see entire document.	1-50

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